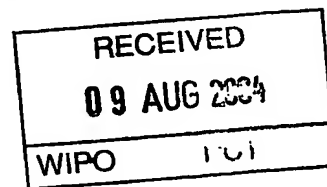




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Archaeon expression system

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**Archaeon expression system**

The present invention relates to a sulfolobus expression vector comprising: (a) sulfolobus origin of replication; (b) the genes encoding the structural proteins and the site-specific integrase of SSV1, SSV2 or pSSVx, operatively linked to expression control sequences and a packaging signal; (c) one or more selectable  
10 marker gene(s), operatively linked to sulfolobus expression control sequences; and (d) a sulfolobus promoter followed 3' by a restriction enzyme recognition site or a multiple cloning site for insertion of a gene of interest and optionally a 3' regulatory element. Moreover, the present invention relates to a shuttle vector comprising the sequences of the expression vector of the invention and additional sequences for  
15 propagation and selection in E. coli, wherein the additional sequences comprise (a) an E.coli ori of replication; and (b) a marker for selection in E.coli. Furthermore, the invention relates to host cells transformed with the expression vector as well as to a kit comprising a vector or a host cell of the present invention. Finally, the present application also relates to a method for generating infectious subviral particles.

20 Several documents are cited throughout the text of this specification. The disclosure content of the documents cited herein (including any manufacture's specifications, instructions, etc.) is herewith incorporated by reference.

Sulfolobus solfataricus is one of the best studied species of hyperthermophilic organisms. It belongs to the Crenarchaeota, one of the two major phyla of the  
25 domain archaea which are almost exclusively represented by hyperthermophiles. Despite its extreme growth requirements (temperature optimum of 78 °C, pH optimum of 3) Sulfolobus can be relatively easily grown under aerobic and heterotrophic conditions in liquid culture and on plates. Due to its ease of cultivation several pioneering studies on archaeal metabolism have been done with Sulfolobus  
30 solfataricus and its relatives (e.g. Bouthier de la Tour et al. 1990, Grogan and Gunsalus 1993, Schafer 1996, Edgell et al. 1997, Elferink et al. 2001, Vitagliano et al. 2001, Wadsworth and White 2001). Furthermore, in vitro studies of transcription, translation and replication in Sulfolobus have provided important insights into the fundamentals of information processing in archaea (Condo et al. 1999, De Felice et  
35 al. 1999, Bell et al. 2001, Hjort and Bernander 2001).

5 With the help of an in vitro transcription system it has been demonstrated, that the basal transcription apparatus of archaea is similar to that of eukaryotes, albeit simpler in composition (reviewed in Bell and Jackson 2001). In brief, the single, rather complex RNA polymerase in archaea, together with a TATA-binding protein (TBP) and TFB, both homologs of the eukaryal transcription factors TBP and TFIIB, 10 respectively, are sufficient to direct accurate initiation of transcription on promoters that contain a TATA-box and BRE element reminiscent of RNA-polymerase II promoters of eukaryotes.

While basic transcription and some regulators have been well studied in vitro, it has not been possible to analyse gene regulation in vivo in hyperthermophiles due to the 15 lack of a powerful genetic system. Efficient genetic systems including reporter genes have only been established for non-thermophilic, halophilic archaea (Patenge et al. 2000, Gregor and Pfeifer 2001). The complete genome sequence of *S. solfataricus* has been determined (She et al. 2001) and a number of genetic elements, such as viruses, conjugative plasmids and small high-copy-plasmids have been 20 characterized and sequenced (for review see Zillig et al. 1998). These elements have served to develop the first genetic tools for *Sulfolobus*, including a transformation/transfection procedure and shuttle vectors (Schleper et al. 1992, Schleper et al. 1995, Aagaard et al. 1996, Elferink et al. 1996, Aravalli and Garrett 1997, Cannio et al. 1998). However, none of the systems described so far have 25 been useful for efficient cloning in *Sulfolobus*, for the high-level expression of proteins or for systematic tests of promoter activities based on a reporter gene. Difficulties were apparently due to low transformation efficiencies, inefficient selection and/or instability of the vectors in the host.

Thus and in of the above, the technical problem underlying the present invention 30 was to provide a genetically stable and highly efficient cloning and expression system for *Sulfolobus*. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly the present invention relates to a *sulfolobus* expression vector comprising (a) a *sulfolobus* origin of replication; (b) the genes encoding the 35 structural proteins and the site-specific integrase of SSV1, SSV2 or pSSVx,

- 5 operatively linked to expression control sequences and a packaging signal; (c) one or more selectable marker gene(s), operatively linked to *sulfolobus* expression control sequences; and (d) a *sulfolobus* promoter followed 3' by a restriction enzyme recognition site or a multiple cloning site for insertion of a gene of interest and optionally a 3' regulatory element.
- 10 The term "*sulfolobus*" refers to the hyperthermophilic Archaeon genera *Sulfolobus* and comprises the species *Sulfolobus acidocaldarius*, *Sulfolobus brierleyi*, *Sulfolobus hakonensis*, *Sulfolobus metallicus*, *Sulfolobus shibatae*, *Sulfolobus solfataricus*. The terms "origin of replication", "ori of replication" or "ori" refer to a region of DNA that is essential for starting its replication.
- 15 The terms "site-specific integrase" or "site-specific recombinase" refer to an integrase molecule which is capable of catalysing the integration of DNA into a specific site in the host chromosome. The attachment site for insertion can be located within the coding sequence of the integrase gene of the expression vector as in the case of SSV1 (vide infra) or outside in the remaining nucleotide sequences
- 20 of the expression vector. Preferably, the integrase is obtainable from the DNA of SSV1 or SSV2, however, any integrase molecule would be comprised by the present invention as long as it catalyses the integration of the expression vector into the chromosomal DNA of the host.

25 The terms "SSV1" and "SSV2" refer to types 1 and 2 of *Sulfolobus shibatae*, a circular double stranded DNA virus adapted to *Sulfolobus*. The term "expression control sequence" refers to elements on the DNA or RNA that control gene expression and therefore include elements such as promoter, enhancer, silencer, transcription factor B recognition element (BRE), Shine Dalgarno sequence, TATA-box, internal ribosomal entry sites (IRES), attachment sites for transcription factors,

30 sequences important for termination of transcription, polyadenylation sites, RNA transporting signals, sequences important for UV-light or heat-shock response causing either gene induction or repression, signal sequences for secretion, splicing signals or nucleotide sequence elements known to be important for intracellular transport, subcellular localization or translation of the RNA. The term "3' regulatory

35 element preferable refers to a polyadenylation sequence. The term "operatively

5 linked" means that a single or a combination of the above-described control elements together with the coding sequence of the gene are capable of directing protein expression from the gene. The term "packaging signal" refers to a region on the viral nucleic genome capable of directing the attached nucleic acid molecules into the envelope of the virion. Although the specific location of the packaging signal is presently unknown, the packaging signal is likely to comprise a region of the DNA capable of interacting with the protein encoded by SSV1 ORF a153 and/or b251 or by the homologous ORF of SSV2. In a particularly preferred embodiment of the present invention, the packaging signal is located within the region encoding SSV1 ORF a153 and/or b251 or with the homologous ORF of SSV2. The term "selectable marker gene" refers to proteins that, when expressed in a host cell, confer a phenotype onto the cell which allows a selection of the cell expressing said selectable marker gene. Generally this may be a protein that confers resistance to an antibiotic such as ampicillin, kanamycin, chloramphenicol, tetracyclin, hygromycin, neomycin or methotrexate. Further examples of antibiotics are Penicillins: Ampicillin HCl, Ampicillin Na, Amoxycillin Na, Carbenicillin disodium, Penicillin G, Cephalosporins, Cefotaxim Na, Cefalexin HCl, Vancomycin, Cycloserine. Other examples include Bacteriostatic Inhibitors such as: Chloramphenicol, Erythromycin, Lincomycin, Tetracyclin, Spectinomycin sulfate, Clindamycin HCl, Chlortetracycline HCl. Additional examples are proteins that allow selection with Bacteriosidal inhibitors such as those affecting protein synthesis irreversibly causing cell death. Aminoglycosides can be inactivated by enzymes such as NPT II which phosphorylates 3'-OH present on kanamycin, thus inactivating this antibiotic. Some aminoglycoside modifying enzymes acetylate the compound and block their entry in to the cell. Gentamycin, Hygromycin B, Kanamycin, Neomycin, Streptomycin, G418, Tobramycin Nucleic Acid Metabolism Inhibitors, Rifampicin, Mitomycin C, Nalidixic acid, Doxorubicin HCl, 5-Fluorouracil, 6-Mercaptopurine, Antimetabolites, Miconazole, Trimethoprim, Methotrexate, Metronidazole, Sulfametoxazole.

The term "Sulfolobus promoter" relates to the promoters of the Sulfolobus host cell wherein the expression vector is suppose to be used in. Preferable the promoters include the promoters of the following ORFs: tf55alpha, tf55beta, tf55gamma, lacS,

5  $T_{ind}$ , SSV1-integrase, pyrEF, 16S ribosomal gene, UV-/heat-shock-/ or otherwise inducible promoters of known, unknown or hypothetical proteins. In addition, the nucleotide sequences of the promoter may contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or up to 15 mutations as long as these mutations do not abrogate the nucleotide sequence's function as an inducible promoter. The term "restriction

10 enzyme recognition site" refers to a motif on the DNA recognized by a restriction enzyme. Preferable the restriction enzyme and its recognition site are selected from the group consisting of AatII, AccI, Acc65I, Acil, AclI, AfeI, AflII, AflIII, AgeI, AhdI, AluI, AlwI, AlwNI, ApaI, ApaLI, Apol, AscI, Asel, AsiSI, Aval, Avall, AvrII, BaeI, BamHI, BanI, BanII, BbsI, BbvI, BbvCI, BceAI, BcgI, BciVI, BclI, Bfal, BfrBI, BfuAI,

15 BglI, BglII, BplI, Bme1580I, BmgBI, Bmrl, BpmI, BsaI, BsaAI, BsaBI, BsaHI, BsaJI, BsaWI, BsaXI, BseRI, BsgI, BsiEI, BsiHKAI, BsiWI, BslI, BsmI, BsmAI, BsmBI, BsmFI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, Bsrl, BsrBI, BsrDI, BsrFI, BsrGI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstF5I, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtrI, BtsI, Cac8I, ClaI, DdeI, DpnI, DpnII, DraI,

20 DraIII, DrdI, EaeI, EagI, EarI, Ecil, EcoNI, EcoO109I, EcoRI, EcoRV, Faul, Fnu4HI, FokI, FseI, FspI, HaeII, HaeIII, HgaI, HhaI, HinP1I, HincII, HindIII, HinfI, HpaI, HpaII, HphI, Hpy99I, Hpy188I, Hpy188III, HpyCH4III, HpyCH4IV, HpyCH4V, KasI, KpnI, MboI, MbolI, MfeI, MluI, MlyI, MnlI, MscI, MseI, MslI, MspI, MspA1I, MwoI, NaeI, NarI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NotI, NruI, NsiI, NspI, PacI,

25 PaeR7I, PciI, PfiFI, PfiMI, Piel, Pmel, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PstI, PvuI, PvulI, RsaI, RsrII, SacI, SacII, Sall, SapI, Sau96I, Sau3AI, SbfI, Scal, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, Smal, SmlI, SnaBI, SpeI, SphI, SspI, Stul, Styl, Swal, TaqI, TfiI, TliI, TseI, Tsp45I, Tsp509I, TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI and XmnI. The term "multiple cloning site" refers to an array of two and

30 more of the above-listed restriction enzyme recognition sites and also includes other recognition sites known to the person skilled in the art which are not listed here.

The vector of the present invention allows to successfully and with high efficiency transform *Sulfolobus* cells, which are a model organism for hyperthermophilic Crenarchaeota. The combination with viral components and a virus-based mode of

35 DNA transfer permits to reach cells, after the initial transformation event, by a process of infection, thereby resulting in a dramatically increased efficiency of

5 transformation. This is achieved by expressing virus structural proteins that are capable of interacting with the packaging signal of the virus and, hence, of specifically packaging DNA attached to the packaging signal. The released viral particles contain a more or less "normal" viral coat, filled with the DNA of the expression vector. The released particles can subsequently enter new cells by  
10 infection which is generally much more efficient than the process of transformation. The presence of two origins of replication allows to propagate the vector both in *E.coli* and in *Sulfolobus*. The marker genes, permit selection of successfully transformed cells, stably harbouring the DNA of the expression vector. Since the expression vector of the present invention also provides a reported gene, successful  
15 transformation can conveniently be monitored by determining the expression from said reporter gene. As the reporter function is available shortly after transformation, time consuming DNA-preparation and Southern Blot analyses are not required. In addition, the presence of a selection marker and of the reporter function permit a straightforward approach to the identification and selection of interesting clones.

20 In a preferred embodiment of the invention, the expression vector contains a *Sulfolobus* origin of replication which is selected from the group consisting of SSV1, SSV2, pSSVx and pRN plasmids (Palm et al., 1991; Schleper et al., 1992; Keeling et al., 1996; Zillig et al., 1994; Arnold et al., 1999).

The plasmids pRN1 and pRN2 amongst others belong to one family of plasmids  
25 (Keeling et al., 1996; Zillig et al., 1994; Arnold et al., 1999). The name pRN refers to the natural host, *Sulfolobus islandicus* RN1H1.

The plasmid pSSVx is defined as a hybrid between a plasmid and a fusellovirus. This plasmid, in the presence of a helper (SSV1 or SSV2) is able to spread as a virus satellite via virus-like particles. Like pRN1 and pRN2, pSSVx belongs to the  
30 pRN family of *Sulfolobus* plasmids, as judged by its genome organization, by the high sequence similarity of a cluster of ORFs and two putative replication origins that comprise 50%-70% of their genomes. However, a tandem array of two ORFs in a non-conserved region in pSSVx is clearly homologous to a similar tandem of ORFs of as yet unknown function in SSV2 and SSV1, suggesting a viral origin for  
35 these plasmid ORFs. The plasmids pRN1 and pRN2, which lack these ORFs, do not



5 spread with the help of SSV1 or SSV2, indicating that a sequence element in this cluster is essential for the packaging and spreading of pSSVx.

The vector of the present invention contains a *Sulfolobus* promoter followed 3' by a restriction enzyme recognition site or a multiple cloning site for insertion of a gene of interest and optionally a 3' regulatory element. Preferentially, the vector contains an attachment site for insertion of a gene of interest. In that case, the gene of interest is preferentially inserted by homologous recombination. The term "attachment site" refers to a sequence either important for recombination of the virus, or its derivatives, into the host chromosome. In particular, the term "attachment site" also refers to artificially added DNA stretches which may flank both 5' and 3' ends of a gene of interest, promoter, selectable marker, termination sequences or sequence tags, amongst others, within the vector of the present invention which are used for targeted exchange by in vitro recombination with another sequence of interest flanked by homologous attachment sequences.

In another preferred embodiment of the invention, the expression vector contains the complete genome of SSV1 or SSV2 or chimeras thereof, thereby providing the *Sulfolobus* origin of replication, the packaging signal and the genes encoding the structural proteins VP1 and VP3 and the integrase of SSV1 or SSV2. In another preferred embodiment of the invention, the expression vector contains the genome of SSV1 or SSV2, wherein the genome contains deletions, substitutions or mutations. Preferable, the depleted viral genome contains in addition to the structural proteins and the integrase gene at least one open reading frame selected from the group consisting of a100, a132, c80, a79, a45, c102b, b129, a291, c124, c792, b78, c166, b115, a82, a84, a92, b277, a153, b251, d335, d244, e178, f93, d63, b49 and e51 (Stedman et al., 2003). All other ORFs occur in both genomes of SSV1 and SSV2.

In yet another preferred embodiment of the invention, the selectable marker gene of the expression vector encodes an essential protein of *Sulfolobus*. In a more preferred embodiment of the present invention, the essential gene is a gene of the de novo nucleotide anabolism, a gene of the aminoacid biosynthesis or a gene conferring antibiotic resistance. In another more preferred embodiment, the vector

5 contains orotidine-5'-monophosphatase pyrophosphorlyase and orotidine-5'-monophosphatase decarboxylase (pyrEF) as selectable marker genes (Martusewitsch et al. 2000).

In a preferred embodiment of the invention, the expression vector contains 3' to the translation initiation site of the promoter for the expression of the gene of interest  
10 additional nucleic acid sequences so that the expressed protein has an N-terminal extension. In a more preferred embodiment, the N-terminal extension is (a) a signal sequence directing the secretion of the expressed protein; or (b) a tag for purification; or (c) a tag for specific detection. The sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a  
15 peptide which facilitates purification of the fused polypeptide or which provides a signal for intracellular transport of the protein. Accordingly, the polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added  
20 to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and  
25 to facilitate purification, among others, are familiar and routine techniques in the art. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), for instance, hexa-histidine provides for convenient  
30 purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37: 767 (1984).

35 In another preferred embodiment, the promoter for the expression of the gene of interest is a constitutive promoter selected from the group consisting of genes

- 5 Involved in central metabolisms and information processing such as the promoters of the ribosomal subunits 16S, 23S rRNA or the promoters of polymerases, transcription, replication or translation factors.

In a further preferred embodiment of the invention, the promoter for the expression of the gene of interest is an inducible promoter and may be selected from the group  
10 consisting of (a) heat inducible promoters Tf55alpha, TF55beta, TF55gamma, hsp20, htrA, (b) cold inducible promoters TF55gamma and (c) promoters inducible by a carbon source such as arabinase, trehalose, maltose, galactose, sucrose.

In yet a further preferred embodiment of the invention the vector contains an additional expression cassette for a reporter protein, selected from the group  
15 consisting of  $\beta$ -galactosidase, luciferase, green fluorescent protein and variants thereof.

The present invention also relates to a shuttle vector comprising the sequences of the expression vector of the invention and additional sequences for propagation and selection in E. coli, wherein the additional sequences comprise (a) an E.coli ori of  
20 replication; and (b) a marker for selection in E.coli. In a preferred embodiment of the invention, the marker of selection is a protein that confers resistance to selected from the group consisting of ampicillin, kanamycin, chloramphenicol, tetracyclin, hygromycin, neomycin or methotrexate.

The present invention also relates to a host cell transformed with the expression  
25 vector of the invention, wherein the host cell is E. coli or sulfolobus. In a preferred embodiment of the invention, Sulfolobus comprises the species Sulfolobus acidocaldarius, Sulfolobus brierleyi, Sulfolobus hakonensis, Sulfolobus metallicus, Sulfolobus shibatae, Sulfolobus solfataricus. Preferred E.coli strains are BL21, BL21(DE3) or BL21(DE3)pLysS and BL21 derivatives, HB101, JM109 and  
30 derivatives, XL-1blue and derivatives, DH10B, DH12S, DH5alpha and derivatives, DB3.1, Stb14, TOP10 and derivatives.

In a preferred embodiment of the invention the transformed expression vector provides a gene encoding an essential protein. In a more preferred embodiment the essential gene is a gene of the de novo nucleotide anabolism, a gene of the

5 aminoacid biosynthesis or a gene conferring antibiotic resistance. In another more preferred embodiment, the essential genes are orotidine-5'-monophosphatase pyrophosphorlyase and/or orotidine-5'-monophosphatase decarboxylase. In another preferred embodiment of the invention, the host is deficient in expressing a fully functional version of said essential gene provided by the expression vector. As a  
10 consequence, the host cell can only survive if the essential protein is provided by expression from the expression vector.

The present invention also relates to a method of producing a polypeptide, comprising culturing the host cell of the present invention under suitable conditions and isolating said polypeptide from the cells or the cell culture supernatant.

15 The present invention also relates to a method of generating infection recombinant subviral particles composed of SSVx or derivatives thereof, wherein SSVx is propagated with the help of a complete virus SSV1 or SSV2. In a preferred embodiment, the methods of the present invention comprises generating infectious recombinant subviral particles composed of the structural proteins of SSV1 and/or  
20 SSV2, having packaged the DNA of the expression vector of the present invention, wherein the method has the steps of (a) introducing the DNA of the expression vector and the DNA of SSV1 or SSV2 into a host cells; (b) incubating the cells for time and under conditions sufficient to allow replication of SSV1 or SSV2 and spreading in the cell culture; (c) harvesting the cell culture supernatant or the host  
25 cells.

The present invention also relates to the use of the vector of the present invention for expression of RNAi or antisense RNA, wherein Sulfolobus promoters of the vector are used for transcription of a gene or parts of a gene either in antisense or sense orientation or in both orientations. In a preferred embodiment of the present  
30 invention's use, the expression vector is used in gene silencing, comprising gene silencing by antisense RNA or gene silencing by double stranded RNA (RNA<sub>i</sub>).

Finally, the present invention relates to a kit comprising (a) the vector of the present invention, (b) the host cell of the present invention, and/or (c) a host cell deficient in the expression of the essential protein, in one or more containers.

5 The figures show:

Figure 1: The figure shows a map of pMJ02a (A) and of pMJ03bx (B), highlighting some recognition sites for restriction enzyme and the relative positions of the pUC18 region, of the reported gene *lacS* and of the selection marker *pyrEF*.

10 Figure 2: Detection of successful transformation with the expression vector by using an X-Gal assay. Cells in solution (A) or cells grown on gelrite plates (B) are shown.

15 Figure 3: Schematic representation by Southern analysis of the extent of transformation of the primary transformation mixture with a recombinant plasmid and of the integration of the recombinant vector into the chromosome of *Sulfolobus solfataricus*.

Figure 4: Specific beta-galactosidase activities in transformants and wild-type cells of *Sulfolobus solfataricus* after heat shock at 88°C.

20 Figure 5: Representative Northern analysis for the detection of the *lacS* transcript after 0 min (0), 30 min (1), 90 min (2) and 22 h (3) of heat shock and 17 h after UV induction (4). Equal amounts (5µg) of total RNA from transformants T1, T2, T3, from the wild type (WT) and from the recipient (R) were separated on a 1,2% agarose gel and hybridized with a specific *lacS* probe. The  
25 detected fragment size is 1,6 kb.

Figure 6: Isolation of total DNA from heat-induced and not heat-induced cultures. Hybridization with SSV1- and/or *lacS*-specific probes (Fig. 6A, B). Correlation of detected amounts of DNA with results of activity assays or Northern analyses, respectively, for the  
30 exclusion of a gene dosage effect.

5 The examples illustrate the invention:

### Example 1: Construction of the Shuttle-Vectors pMJ02a and pMJ03bX

For the construction of pMJ02a (Figure 1A), the 1,5 kbp *lacS* ORF was PCR amplified from chromosomal DNA of *S. solfataricus* P1, using the primer LacS2.F  
 10 (GCTCCAGTCATGTACTCATTTCCAAATAGC) and LacS.R-Eag (GAAACGGCCGGCAATCTAATG). The promotor region, including the first 5 codons of the open reading frame of *tf55α* were also PCR amplified from chromosomal DNA of *S. solfataricus* P1, using the primers TF55prom.F-Eag (ATTAAGTCGGCCGTCAAGAAA) and TF55prom2.R  
 15 (TGAGTACATGACTGGAGCTGCCATACC). Both PCR products obtained were used in a second PCR-reaction, using the primers TF55prom.F-Eag and LacS.R-Eag. By using overlapping complementary sequences of TF55prom2.R and LacS2.F in this second PCR reaction, the TF55α-Promotor was fused to the *lacS*-ORF and was subsequently amplified. The resulting 2055 bp PCR product was  
 20 cleaved by *EagI* and cloned into pBluescriptSK+. After sequence analysis, the fragment was isolated from the vector as a *PstI* (dephosphorylated) and *SacI* fragment and ligated to pUC18 (*PstI*/*SacI* and dephosphorylated) and SSV1 (*PstI*) in a ligation of three fragments .

pMJ03bX (Figure 1B) was obtained by PCR-amplifikation of the *pyrEF* Gene using  
 25 chromosomal DNA of *S. solfataricus* P1 and the primers pyrEF.F-Nhe (TCTCGCTAGCGAATAATGCTGCCC) and pyrEF.R-NheI (TTACGCTAGCTTCCTCGTGTAGAT) and ligation, after *NheI*-cleavage, into pMJ02a (*XbaI*, dephosphorylated). After electroporation of *E. coli* DH10B positive clones were identified by colony hybridisation with a SSV1-specific probe and a  
 30 *lacS*-specific probe. The plasmids isolated from *E. coli* were characterized by restriction analysis and the orientation of the insert was determined.

## 5 Example 2: Transformation of *Sulfolobus*

Electroporation was performed under optimized conditions according to a previously established protocol for *Sulfolobus* (Schleper, 1992). Electroporation-competent cells are prepared from a 50 ml over night culture ( $OD_{600nm} = 0,1$  to  $0,3$ ). The cells are cooled on ice and centrifuged for 15 min at 4.000 rpm and  $4^{\circ}C$ . The cell pellet is subsequently washed with 50, 25 and 1 ml of pre-cooled 20 mM sucrose to remove salts from the cell culture medium. Using 20 mM sucrose, the cells are adjusted to a concentration of  $10^{10}$  cells/ml. Prior to electroporation, the competent cells are kept on ice. 50  $\mu$ l of cell suspension are carefully mixed with 0,25 to 1,5  $\mu$ l of a dialyzed plasmid-DNA suspension (max. 300 ng DNA) and transferred to a pre-cooled electroporation cuvette (distance of electrode: 0,1 cm, BIO-RAD). Electroporation is performed by using a Gene Pulser (Bio-Rad), using the following settings:

20 Voltage 1,5 kV  
Capacitiy 25  $\mu$ F  
Resistance 400  $\Omega$

Immediately after electroporation the cells are transferred to 1 ml growth media, in 1,5 ml reaction tube, and shaken for 1 to 2 h on a heating plate at  $75^{\circ}C$ , with occasional aeration. Subsequently, the transformed cells are transferred to 50 ml of medium.

25

## Example 3: Detection of transformants in the primary transformation mixture and at the level of single transformants

After the transformation of *Sulfolobus* mutants, transformants could be detected qualitatively already in the transformation mixture approx. 3 days after electroporation. A blue stain obtained after incubation at  $78^{\circ}C$  of a culture aliquot (approx. 800 $\mu$ l) with 100 $\mu$ l X-Gal (5mg/ml) indicates successful transformation, the blue staining resulting from cleavage of X-Gal and release of the chromophor (Fig. 2A). Single transformants can reliably be identified and isolated by their blue stain

- 5 after adding X-Gal to single colonies grown from primary transformation mixtures on Gelrite plates and, if necessary, incubating them at 78°C (Fig. 2B).

The initial extent of transformation of the primary transformation mixture with the recombinant plasmid and the chromosomal integration in single transformants are detected by Southern analysis of total DNA isolated from the primary transformation  
 10 mixture and of single transformants and by hybridization with a SSV1-specific probe (Fig. 3).

Schematic representation by Southern analysis of the extent of transformation of the primary transformation mixture with a recombinant plasmid and of the integration of the recombinant vector into the chromosome of *Sulfolobus solfataricus*.

- 15 For the purpose of hybridization with a SSV1-specific probe, total DNA isolated from a primary transformation mixture 3 days (a), 4 days (b) and 5 days (c) after electroporation as well as from single transformants (T1, T2), from the wild type *Sulfolobus solfataricus* P1 and from the recipient (R) was cleaved hydrolytically with *PvuII*. PL designates the recombinant vector isolated from *E. coli*.
- 20 At the beginning, the recombinant virus spreads rapidly in the primary transformation mixture and is therefore detectable only in its plasmid form (lane a, b, c) whereas in isolated single transformants, the virus is detectable only its form as integrated into the chromosome. The 20 kbp and the 7,5 kbp fragment represent the right and left element of the integrated vector, whereas the plasmid-specific 11,3  
 25 band is not any more detectable in single transformants (cf. also Fig. 6).

#### Example 4: Regulation studies

Using the newly developed vector, the regulation of gene expression in hyperthermophile Archaea was analyzed *in vivo*.

- 30 *Analysis of the regulation mechanisms of the TF55 $\alpha$  promoter in case of heat shock induction*



5 Single transformants grown in liquid culture up to an OD 600 nm of 0,2 – 0.3 are incubated for different periods of time (30 min to 46 h) at 88°C. The induction of lacS expression caused by heat shock and performed by the inducible promoter is quantified by subsequent  $\beta$ -galactidose activity assays (Fig. 4). Raw cell extracts are obtained as follows:

10 Cell pellets obtained from 5-10 ml cell culture are re-suspended in approx. 100  $\mu$ l 10 mM Tris/HCl buffer, pH 8 and lysis is performed by freezing at –80°C for 10 min and thawing at 50°C (5 min). This procedure is repeated four times and the obtained extract is centrifuged at approx. 10000 g for 30 min. The supernatant is either used immediately for an enzyme assay or stored at –80°C. The protein concentration of  
15 the raw extracts is measured in a Bradford assay.

The  $\beta$ -galactidose assay is performed according to Pisani et al. (1990). 10  $\mu$ l extract are transferred into a pre-heated quartz cuvette (75°C) with 990  $\mu$ l test buffer (2,8 mM 0-nitrophenyl  $\beta$ -D-galactopyranosid in 50 mM sodium phosphate buffer, pH 6.5). Hydrolysis of ONPG is monitored photometrically by the increase of absorption  
20 at 405 nm. One Unit is defined as the amount of enzyme, which catalyses the hydrolysis of 1  $\mu$ mol ONPG/min at 75°C. The molar absorption coefficient of ONPG at these conditions is 3100 M<sup>-1</sup> x cm<sup>-1</sup>.

*Northern analyses: Detection of induced lacS-mRNA with a lacS-specific probe in heat-induced cultures compared to non-induced cultures.*

25 Total RNA is isolated using the Rneasy kit (Qiagen) and treated subsequently with DNase I. For the purpose of transcript analysis, 5  $\mu$ g RNA are separated on a denaturing agarose containing 1,2 % (w/v) formaldehyde and subsequently transferred to a nylon membrane. A lacS-specific probe is prepared with the T3/T7 system. pBluescript with inserted *tf55-lacS* fusion, hydrolytically cleaved with *Pag* I,  
30 is used as a template. The probe is labeled with digoxigenin (DIG RNA labeling kit, Roche).

Representative Northern analysis for the detection of the *lacS* transcript after 0 min (0), 30 min (1), 90 min (2) and 22 h (3) of heat shock and 17 h after UV induction

- 5 (4). Equal amounts (5µg) of total RNA from transformants T1, T2, T3, from the wild type (WT) and from the recipient (R) were separated on a 1,2% agarose gel and hybridized with a specific *lacS* probe. The detected fragment size is 1,6 kb.

The comparison of transcript amounts in transformants and the wild type shows that the *tf55* promoter, which regulates the recombinant *lacS*, causes an increased transcription of *lacS*, which furthermore is inducible by heat. After a 90 min heat shock a strong increase of *lacS* transcript is observed, which is detectable also after a 22h heat shock.

15 *Control experiment for the exclusion of increased activities or transcript amounts, respectively, caused by a gene dosage effect, using DNA analysis (Southern analysis). Isolation of total DNA from heat-induced cultures and from not heat-induced cultures.*

Isolation of total DNA from *Sulfolobus*:

20 3 ml or 50 ml, respectively, of a culture in the exponential growth phase were pre-cooled on ice for a few minutes and 15 minutes centrifuged at 4000 rpm. The cells are re-suspended in 500 µl or 5 ml, respectively, TE buffer and incubated at room temperature for 30 min after adding N-laurylsarcosin (final concentration 0,8%) and Triton X-100 (final concentration 0,06%).

25 After lysis of the cells, the proteins are extracted three times with a phenol/chloroform/isoamyl alcohol mixture (25:24:1). The DNA is precipitated during 20 min at -20°C after adding 3M sodium acetate solution (1/10 of the volume) and ice cold 100% ethanol (2 to 2,5 volumes). Subsequently, the DNA is centrifuged at 15000 rpm and 4°C for 30 min, washed once with 70% ethanol, air dried and taken up in a suitable volume TE buffer with 10 µg/ml RNase A

30 *Isolation of total from heat-induced and not heat-induced cultures. Hybridization with SSV1- and/or lacS-specific probes (Fig. 6A, B). Correlation of detected amounts of DNA with results of activity assays or Northern analyses, respectively, for the exclusion of a gene dosage effect.*

- 5 Southern analysis of the transformants (T1, T2, T3), of the recipient (R) and of the wild type (WT) after 0 min (0), 30 min (1) and 90 min (2) heat shock. Total DNA was cleaved hydrolytically with *PvuII* and hybridized with a SSV1-specific probe (A) and a *lacS*-specific probe. PL denotes plasmid DNA of the recombinant vector isolated from *E. coli*, hydrolytically cleaved with *PvuII*. It is demonstrated that the heat shock
- 10 does not lead to an increase of recombinant vector and an increase in activity is not due to a gene dosage effect.

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02. Juni 2003

## Claims

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1. A sulfolobus expression vector comprising:

- (a) a sulfolobus origin of replication;
- (b) the genes encoding the structural proteins and the site-specific integrase of SSV1, SSV2 or pSSVx, operatively linked to expression control sequences and a packaging signal;
- (c) one or more selectable marker gene(s), operatively linked to sulfolobus expression control sequences; and
- (d) a sulfolobus promoter followed 3' by a restriction enzyme recognition site or a multiple cloning site for insertion of a gene of interest and optionally a 3' regulatory element.

10

15

2. The expression vector of claim 1, wherein the origin of replication of (a) is selected from the group consisting of SSV1, SSV2, pSSVx and pRN plasmids.

20

3. The expression vector of claim 1 or 2, wherein the vector contains the complete genome of SSV1, thereby providing said origin of replication, said packaging signal and said genes encoding the structural proteins and the integrase of SSV1.

4. The expression vector of any one of claims 1 to 3, wherein the selectable marker gene of (c) encodes an essential protein of sulfolobus.

25

5. The expression vector of claim 4, wherein the essential gene is a gene of the de novo nucleotide anabolism, a gene of the aminoacid biosynthesis or a gene conferring antibiotic resistance.

30

6. The expression vector of claim 4, wherein the vector contains orotidine-5'-monophosphatase pyrophosphorlyase and orotidine-5'-monophosphatase decarboxylase as selectable marker genes.

7. The expression vector of any one of claims 1 to 6, wherein the vector contains 3' to the translation initiation site of the promoter for the expression



- 5 of the gene of interest additional nucleic acid sequences so that the expressed protein has an N-terminal extension.
8. The expression vector of claim 7, wherein the N-terminal extension is
- (a) a signal sequence directing the secretion of the expressed protein;
  - (b) a tag for purification; or
  - 10 (c) a tag for specific detection.
9. The expression vector of any one of claims 1 to 8, wherein the promoter for the expression of the gene of interest is a constitutive promoter selected from the group consisting of genes involved in central metabolisms and information processing including the promoters of the ribosomal subunits
- 15 16S, 23S rRNA or the promoters of polymerases, transcription, replication or translation factors.
10. The expression vector of any one of claims 1 to 9, wherein the promoter for the expression of the gene of interest is an inducible promoter.
11. The expression vector of claim 10, wherein the inducible promoter is selected
- 20 from the group consisting of (a) heat inducible promoters Tf55alpha, TF55beta, TF55gamma, hsp20, htrA, (b) cold inducible promoters TF55gamma and (c) promoters inducible by a carbon source.
12. The expression vector of any one of claims 1 to 11, wherein the vector contains an additional expression cassette for a reporter protein, selected
- 25 from the group consisting of  $\beta$ -galactosidase, luciferase, green fluorescent protein and variants thereof.
13. A shuttle vector comprising the sequences of the expression vector of any one of claims 1 to 12 and additional sequences for propagation and selection in E. coli, wherein the additional sequences comprise
- 30 (a) an E.coli ori of replication; and
- (b) a marker for selection in E.coli.

- 5 14. The shuttle vector of claim 13, wherein the marker of selection is selected from the group consisting of ampicillin, kanamycin, chloramphenicol, tetracyclin, hygromycin, neomycin or methotrexate.
15. A host cell transformed with the expression vector of any one of claims 1 to 14, wherein the host cell is *E. coli* or *sulfolobus*.
- 10 16. The host cell of claim 15, wherein the transformed expression vector provides a gene encoding an essential protein.
17. The host cell of claim 15, wherein the host is deficient in expressing a fully functional version of said essential gene provided by the expression vector.
- 15 18. A method of producing a polypeptide comprising culturing the host cell of any one of claims 15 to 18 under suitable conditions and isolating said (poly)peptide from the cells or the cell culture supernatant.
19. A method of generating infectious recombinant subviral particles composed of the structural proteins of SSV1 and/or SSV2, having packaged the DNA of the expression vector of any one of claims 1 to 14, wherein the method has the steps of
- 20 (a) introducing the DNA of the expression vector and the DNA of SSV1 or SSV2 into a host cells;
- (b) incubating the cells for time and under conditions sufficient to allow replication of SSV1 or SSV2 and spreading in the cell culture;
- 25 (c) harvesting the cell culture supernatant or the host cells.
20. Use of the vector of any one of claims 1 to 14 for gene silencing by expression of RNAi or antisense RNA, wherein the vector contains a *Sulfolobus* promoter for transcription of a gene or parts of a gene either in antisense or sense orientation or in both orientations.
- 30 21. A kit comprising
- (a) the vector of any one of claims 1 to 14,
- (b) the host cell of any one of claim 15 to 17, and/or

- 5 (c) a host cell deficient in the expression of the essential protein of the vector of (a).  
in one or more containers.

**Abstract**

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The present invention relates to a *sulfolobus* expression vector comprising: (a) *sulfolobus* origin of replication; (b) the genes encoding the structural proteins and the site-specific integrase of SSV1, SSV2 or pSSVx, operatively linked to expression control sequences and a packaging signal; (c) one or more selectable marker gene(s), operatively linked to *sulfolobus* expression control sequences; and (d) a *sulfolobus* promoter followed 3' by a restriction enzyme recognition site or a multiple cloning site for insertion of a gene of interest and optionally a 3' regulatory element. Moreover, the present invention relates to a shuttle vector comprising the sequences of the expression vector of the invention and additional sequences for propagation and selection in *E. coli*, wherein the additional sequences comprise (a) an *E. coli* ori of replication; and (b) a marker for selection in *E. coli*. Furthermore, the invention relates to host cells transformed with the expression vector as well as to a kit comprising a vector or a host cell of the present invention. Finally, the present application also relates to a method for generating infectious subviral particles.

1/6

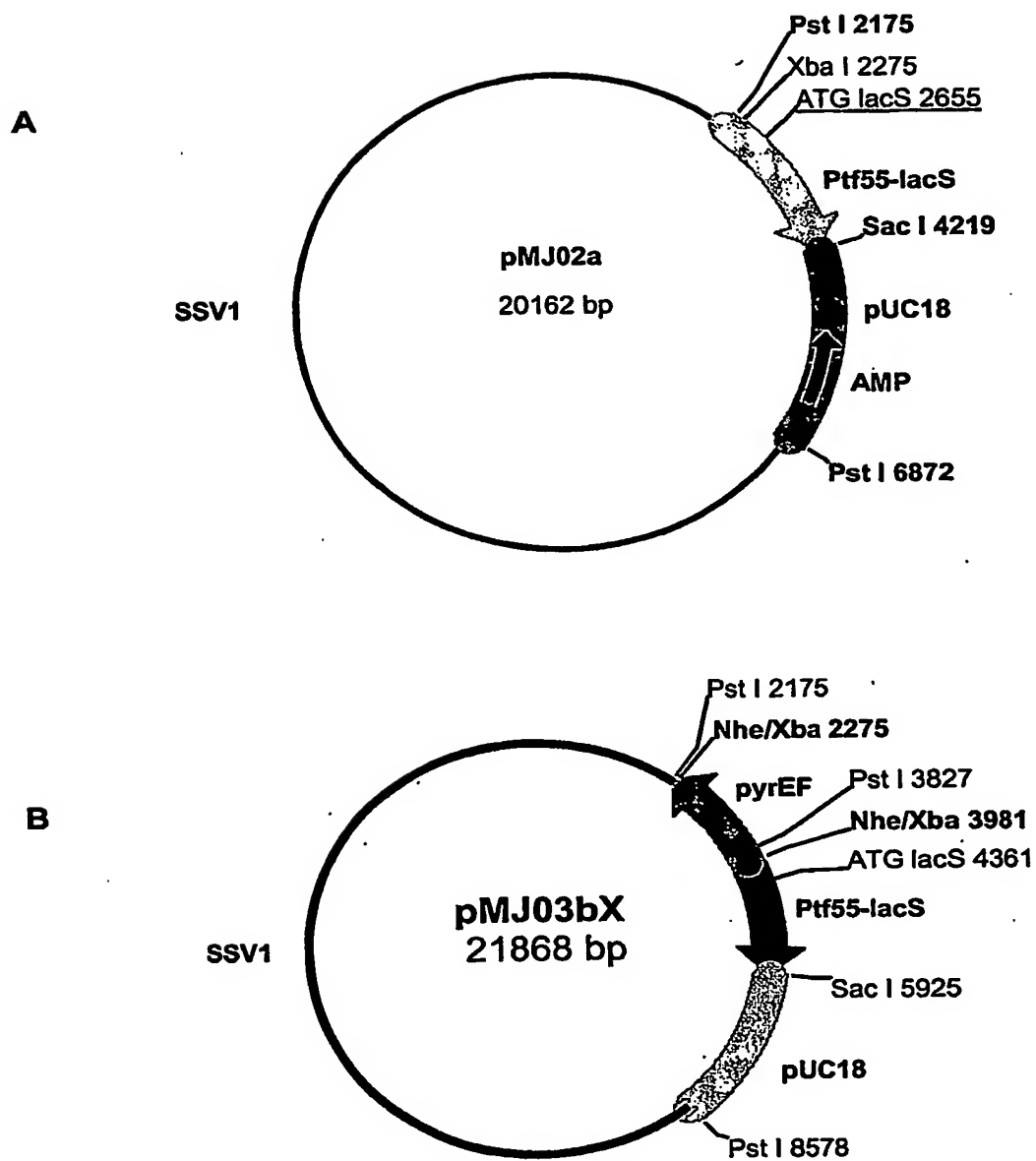


Figure 1

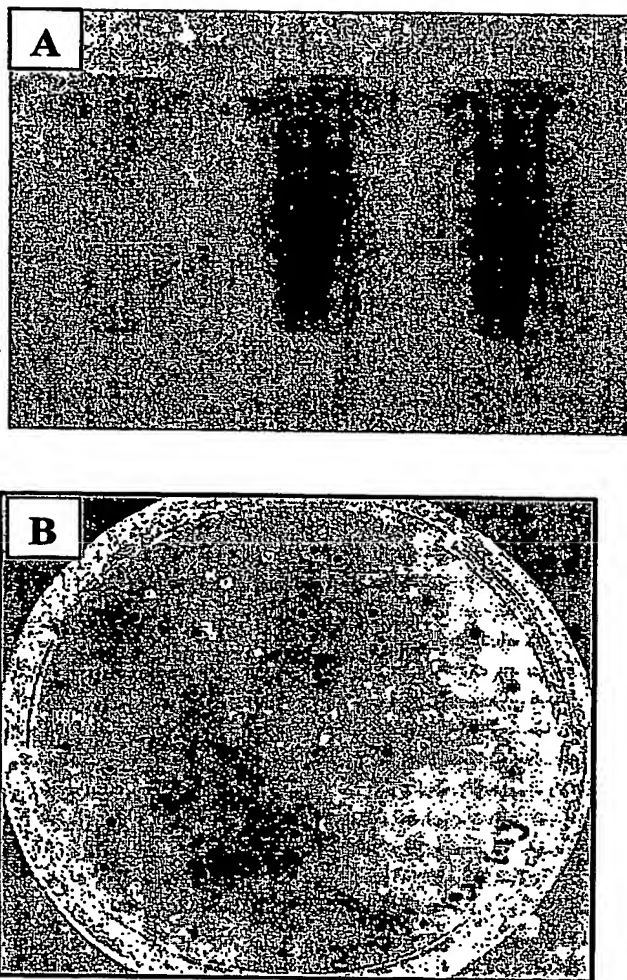


Figure 2

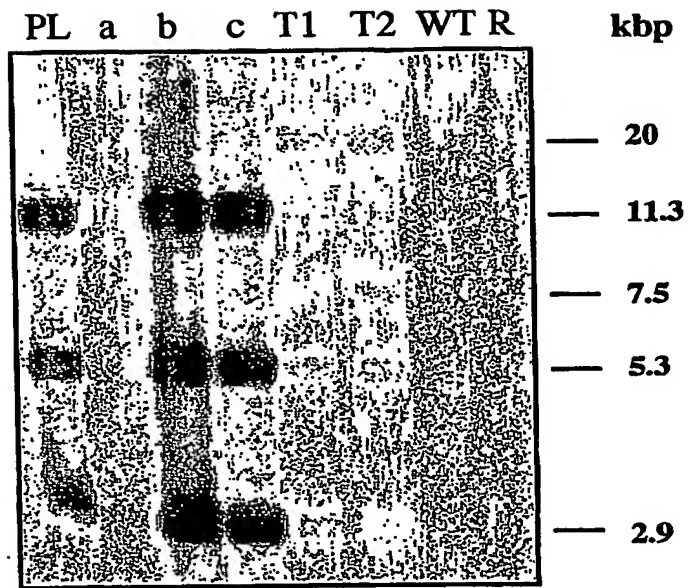


Figure 3

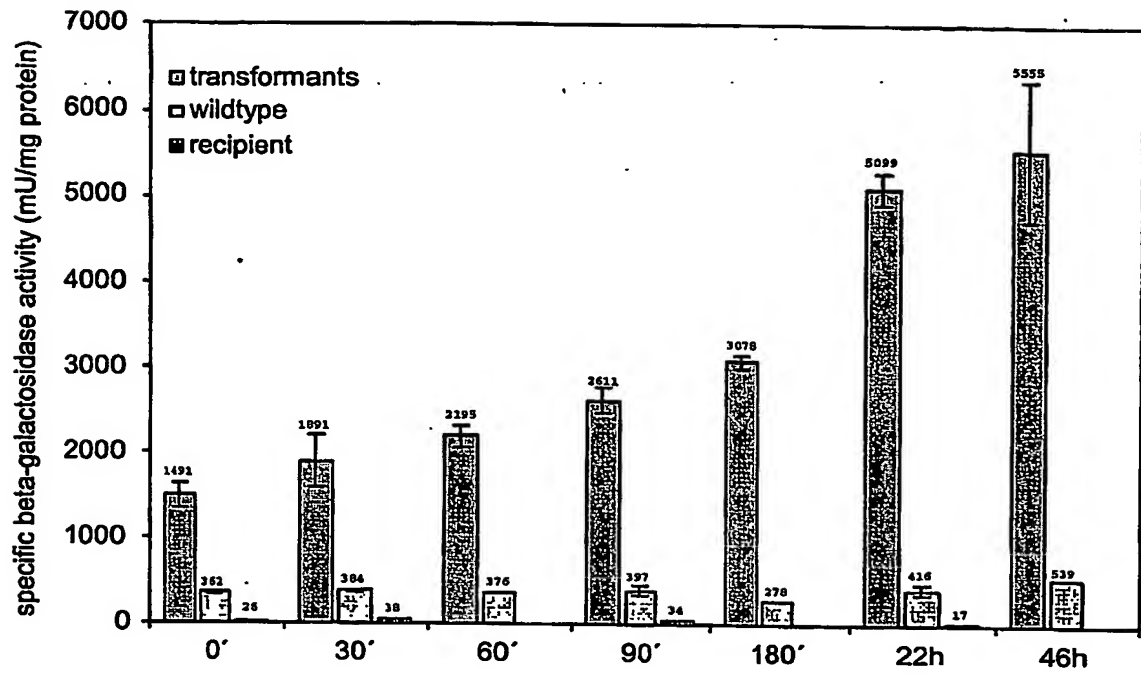


Figure 4



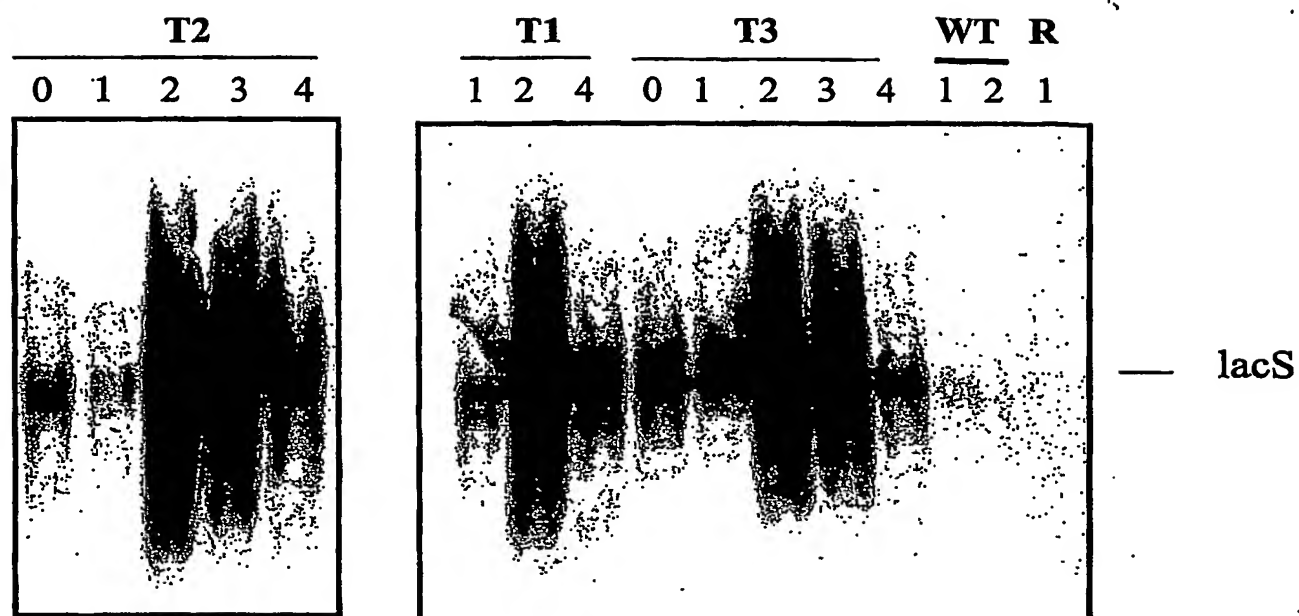


Figure 5

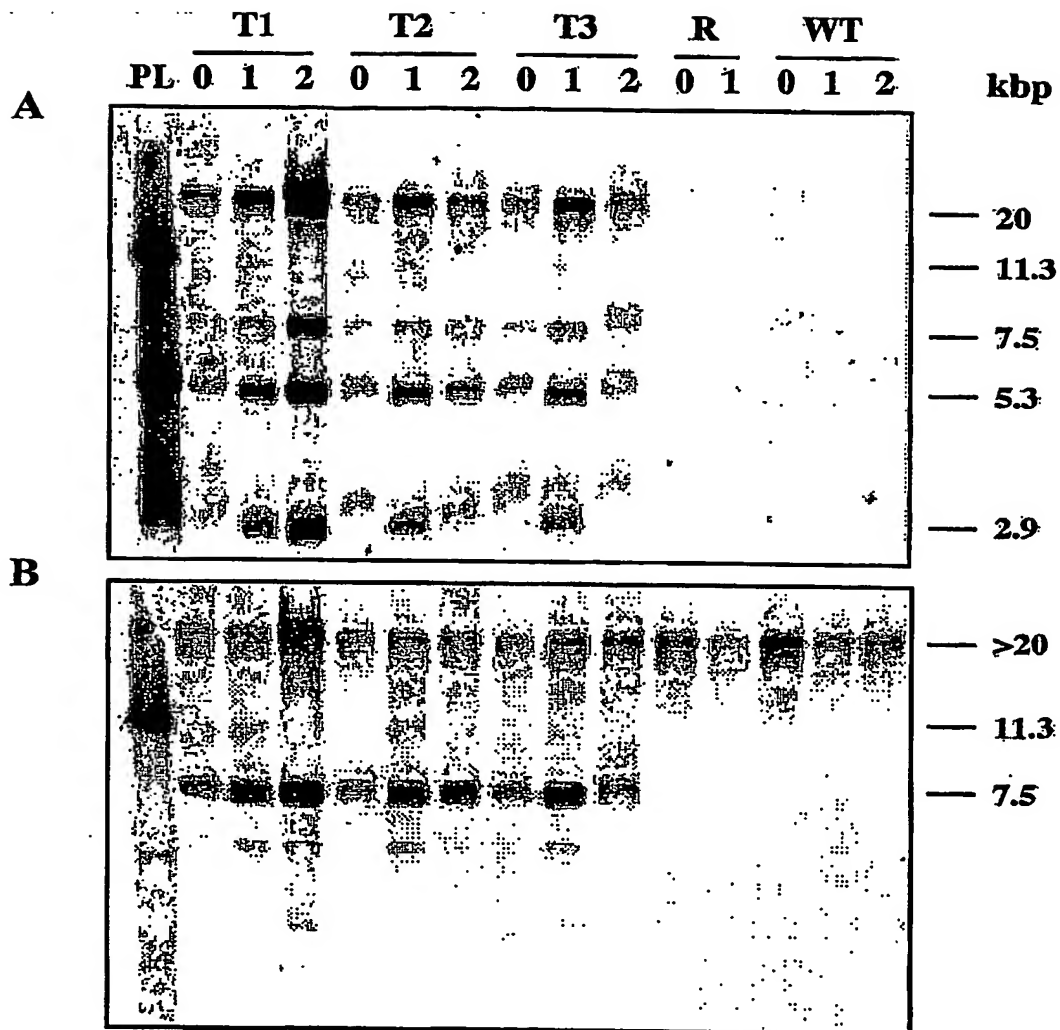


Figure 6